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Original Article

The Role of N-acetylcysteine Amide in Defending Primary Human Retinal Pigment Epithelial Cells against Tert-butyl hydroperoxide-induced Oxidative Stress

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ABSTRACT

Background: Age-related macular degeneration (AMD) is a leading cause of blindness in the United States among adults age 60 and older. While oxidative stress is implicated in the pathogenesis of AMD, dietary antioxidants have been shown to delay AMD progression in clinical studies. We hypothesized that N-acetylcysteine amide (NACA), a thiol antioxidant, would protect retinal pigment epithelium and impede progression of retinal degeneration. **Methods:** *tert*-Butyl hydroperoxide (TBHP) was used to induce oxidative stress in cell cultures. The goal was to evaluate the efficacy of NACA in an *in vitro* model of AMD in primary human retinal pigment epithelial cells (HRPEpiC). **Results:** Our data indicates that TBHP generated reactive oxygen species (ROS), which reduced cell viability, depleted glutathione (GSH) levels, and compromised glutathione reductase (GR) activity. Pretreatment with NACA significantly reduced ROS generation, restored GSH levels and GR activity, and recovered transepithelial electrical resistance. Pretreatment with NACA did not decrease the number of dying cells as determined by flow cytometry analysis. However, survival was significantly improved when cells were co-exposed to NACA and TBHP after a shortened pretreatment period. **Conclusion:** Our data suggest that pretreatment with NACA reduces sublethal but not lethal effects of TBHP in HRPEpiC. NACA significantly improves cell survival when administered prior to and during oxidative damage similar to that observed in the development of dry AMD. These results indicate that continuation of a thiol antioxidant regimen for treatment of AMD is beneficial throughout the course of the disease, and NACA is a potent antioxidant that should be further evaluated for this purpose.

Key words: N-acetylcysteine amide, Thiol antioxidant, Oxidative free radical damage, Retinal pigment epithelium.

Key Messages: NACA defends RPE against oxidative stress in an *in vitro* model of AMD.

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INTRODUCTION

Age-related macular degeneration (AMD) accounts for 8.7% of blindness worldwide, and it is a leading cause of blindness in the United States among adults age 60 and older.[1-3] Globally, about 1 in 32 visual impairments and 1 in 15 cases of blindness are due to macular disease.[4] Dry AMD is a degenerative condition that begins in Bruch's membrane and progresses to the retinal pigment epithelium (RPE) and the overlying photoreceptors with loss of central vision. The RPE is a single layer of pigment cells lying between the photoreceptors and choriocapillaris. The functions of the RPE include light absorption, epithelial transport, phagocytosis, secretion, and immune modulation. All of these functions are important to maintaining and supporting the photoreceptors. High blood flow rate and high O_2 saturation in the choriocapillaris render the RPE more exposed to oxidative stress.[5]

Oxidative stress seems to play a major role in the pathogenesis of dry AMD and retinal degeneration, although the pathophysiology of dry AMD is complex.[5,6] The macula is subject to increased levels of photooxidative stress because of its elevated metabolic rate and high proportion of polyunsaturated fatty acids, which are highly susceptible to lipid peroxidation. As the RPE cells of the macula age, oxidation of lipids and other cellular components results in an accumulation of indigestible lipofuscin in the lysosomes. The accumulation of lipofuscin granules closely parallels drusen formation in time course and distribution in the retina. Oxidative damage to the mitochondrial DNA in RPE cells has also been implicated in the development of AMD. Levels of antioxidants in retinal tissue, in particular glutathione (GSH), decrease with aging, making the macula even more vulnerable to progressive oxidative damage.[7,8]

Endogenous antioxidants, such as GSH, cannot be replaced directly. Instead, compounds that can easily enter cells and increase intracellular antioxidant levels are preferred. Dietary antioxidants have been shown to slow the progression of retinal degeneration and halt progression of AMD in human clinical trials.[9,10] GSH and its amino acid precursors protect RPE cells from oxidative injury and oxidant-induced apoptosis *in vitro*. N-acetylcysteine amide (NACA), a GSH prodrug, is a promising candidate for use as an antioxidant-based treatment for AMD. It has demonstrated efficacy in numerous *in vitro* and animal models of other oxidative stress-related conditions, including drug-induced RPE damage and cataracts.[11-15] Our lab has demonstrated that NACA prevented retinal degeneration *in vitro* and in animal models.[14] The immortalized ARPE-19 cell line was used as an *in vitro* model because primary human RPE cells were not available at the time. ROS generation and lipid peroxidation induced by *tert*-butyl hydroperoxide (TBHP) were reversed by pretreatment with NACA. NACA also protected against TBHP-induced cell damage by increasing cellular levels of GSH, and maintaining cellular integrity, as measured by transepithelial electrical resistance (TEER). In an animal model, phototoxicity-induced photoreceptor cell death and photoreceptor dysfunction were prevented by NACA.[14]

The experiments reported here utilized primary cultures of human retinal pigment epithelial cells (HRPEpiC), the best available *in vitro* model

for studying RPE-related disorders. Because we attempted to mimic a human disease and provide a solution using a "disease-in-the-dish" model, choosing the most representative cell model was critical. Thus, we decided to determine whether the beneficial effects of NACA observed in ARPE-19 cells could also be observed in the HRPEpiC. Our goals were to investigate the following: (1) the sublethal and lethal effects of TBHP in HRPEpiC and (2) the protective effects of NACA pretreatment against TBHP-induced oxidative damage. Sublethal effects included changes in redox status (ROS generation, GSH levels and GR activity) and monolayer integrity (TEER and dextran permeability). Lethal effects were determined with cell viability and apoptosis assays.

MATERIALS AND METHODS

Materials

N-acetylcysteine amide (NACA) was provided by Dr. Glenn Goldstein (David Pharmaceuticals, New York, NY, USA). Human retinal pigment epithelial cells (HRPEpiC) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Other chemicals were ordered from Sigma (St. Louis, MO, USA), unless otherwise indicated.

HRPEpiC culture and experimental design

HRPEpiC were grown in complete media and divided in to 4 groups (control, NACA, TBHP and NACA+TBHP). Cells were pretreated with 2 mM NACA (in NACA and NACA+TBHP groups) or serum-free media (in control and TBHP groups). After 4 h incubation, the media was removed, and followed by dosing with 0.5 mM TBHP for 3 h. For apoptosis assays, an alternative NACA and TBHP dosing protocol was used in addition to the one described above. This involved pretreatment with NACA for 1 h prior to TBHP exposure and addition of NACA for another 3 h (i.e., the NACA+TBHP group cells were co-exposed to TBHP and NACA for 3 h).

Cell viability assay

1×10^5 cells per well were seeded in a 96-well plate for 24 h before further dosing. After cells were treated as indicated in the experimental design, 2 μM Calcein-AM (Biotium Inc., CA) was added to each well, and cells were incubated for 30 min before measuring the fluorescence by plate reader (λ_{ex} = 485 nm and λ_{em} = 530 nm) (FLUOstar Omega, BMG Labtech).

Intracellular ROS measurement

10 μM of CM-H_2DCFDA (Molecular Probes, Life Technologies) was loaded prior to dosing according to the experimental design. Fluorescence was measured after treatment (λ_{ex} = 485 nm and λ_{em} = 530 nm).

Quantification of intracellular glutathione (GSH) levels

Cells were grown at a density of 1.2×10^6 per 10-cm plate for 24 h before further treatment. GSH was separated and quantified by HPLC with fluorescence detection (Finnigan Surveyor FL Plus, Thermo Scientific) after derivatization of cell homogenates with N-(1-pyrenyl)maleimide (NPM).[16]

Glutathione reductase (GR) activity assay

Cells were homogenized in 250 μl of 50 mM Tris-HCl (pH = 7.5) containing 1 mM EDTA. NADPH, which absorbs light at 340 nm, was added to each sample. The rate of its oxidation to $NADP^+$ by GR as GSSG is reduced to 2 GSH can be determined from the resulting decrease in absorbance at this wavelength over time.[17]

Determination of Protein

Protein levels of the cell homogenates were measured by the Bradford method (BioRad Laboratories, Inc., CA, USA). Bovine serum albumin was used as the protein standard.

Dextran permeability and transepithelial electrical resistance (TEER) measurement

12-mm Transwell inserts were coated with poly-L-lysine for 1 h prior to seeding 0.5 ml at 1×10^6 cells/ml. Cell growth was maintained for 5 days without disturbing the monolayer. Fluorescently labelled dextrans (FD4, 1 mg/ml) were administered apically onto the insert after dosing. Fluorescence was read after 30 min (λ_{ex} =485 nm and λ_{em} = 530 nm). The inserts containing the monolayer were then transferred to a new well with serum-free phenol red-free media and used for TEER measurement with an EVOM voltohmmeter (World Precision Instrument, Sarasota, FL, USA) to assess the integrity of HRPEpiC monolayer.

Flow cytometry quantification of apoptotic cells

Cells were grown in 6-cm plates and dosed as described by the experiment design. Trypsinized cells were re-suspended in binding buffer containing 7-AAD and annexin V-FITC (BD Biosciences, San Jose, CA, USA). Cells were incubated for 15 minutes at room temperature in the dark then analyzed by Cell Lab Quanta SC flow cytometry (Beckman Coulter, Fullerton, CA, USA).

Statistical Analysis

All values are reported as mean ± SD (n = 3-10). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Statistical significance was performed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. #, ## and ### represent P<0.05, 0.01 and 0.001, respectively, compared to control; *, ** and *** represent P<0.05, 0.01 and 0.001, respectively, compared to TBHP group.

RESULTS

TBHP increased intracellular ROS levels in HRPEpiC, and NACA prevented ROS generation

To investigate ROS generation in response to TBHP treatment, ROS levels were measured after exposing cells to TBHP for 3 h. TBHP induced ROS generation in a dose-dependent manner (Figure 1A). ROS levels in TBHP-treated cells were 177% of those observed in the control group (P<0.001). Pretreatment with NACA decreased ROS levels to 126% of those observed in the control group (P<0.001 compared to TBHP group) (Figure 1B).

Dose-dependent GSH depletion by TBHP and revention by pretreatment with NACA

To elucidate the consequences of elevated ROS in HRPEpiC, GSH levels were measured after treatment for 3 h with various concentrations of TBHP. TBHP decreased cellular GSH levels in a dose-dependent manner: the average GSH levels in cells treated with 0, 0.5, 1, 25 and 50 mM TBHP were 33.4 ± 5.0, 25.1 ± 1.5, 18.9 ± 0.7, 8.8 ± 1.9 and 6.2 ± 1.3 nmol/mg protein, respectively (P<0.001 compared to control) (Figure 2A). The protective effect of NACA on GSH depletion in cells treated with TBHP was also investigated (Figure 2B). GSH is the major antioxidant in the cell and NACA is a GSH precursor, providing cysteine for GSH synthesis. The average GSH level in the control group was 31.7 ± 2.3 nmol/mg protein. The GSH level was increased after NACA incubation (55.1 ± 7.3 nmol/mg protein, P<0.001 compared to control). The average GSH level

of the TBHP group was 25.1 ± 1.5 nmol/mg protein (P<0.01 compared to control), but the NACA+TBHP group had an average GSH level of 30.5 ± 2.5 nmol/mg protein (P < 0.05, compared to the TBHP group).

Preservation of glutathione reductase activity by pretreatment with NACA

Glutathione reductase (GR), an enzyme which reduces glutathione disulfide to GSH, is important in restoring intracellular levels of GSH. GR activity was reduced in the TBHP group (33.5 ± 3.2 versus 21.9 ± 2.2 mU/mg protein in the control group; P<0.01) (Figure 3). NACA-pretreated cells exhibited increased GR activity (33.5 ± 5.2 mU/mg protein; P<0.01 compared to the TBHP group).

Effects of oxidative stress on HRPEpiC monolayer paracellular permeability and contribution of NACA to monolayer functional homeostasis

RPE paracellular permeability was assessed in HRPEpiC grown on a polycarbonate insert, which form a monolayer similar to that of the blood-retina barrier *in vivo*. Transepithelial electrical resistance (TEER) was used as a measure of paracellular permeability to ions, where lower electrical resistance is evidence of decreased membrane integrity of HRPEpiC in the monolayer. Permeability to non-ionic molecules was determined based on the amount of dextran able to pass through the monolayer within 30 min. Compared to the TBHP group, the NACA+TBHP group exhibited significantly greater electrical resistance (Figure 4A). Dextran permeability in the TBHP group was significantly higher than control (P<0.05) (Figure 4B).

Cell viability and lethal effects of TBHP in HRPEpiC

To investigate cell survival in response to ROS generation, cell viability assays were performed after treatment (Figure 5). Pretreatment with NACA alone did not affect cell viability (91.1 ± 4.5%) compared to control (100.0 ± 10.1%). Cell viability was reduced in the TBHP group (52.2 ± 5.5%, P<0.001). Pretreatment with NACA provided slight but not statistically significant protection against TBHP-induced cell death.

Annexin V-FITC and 7-aminoactinomycin-D (7-AAD) detection of apoptosis by flow cytometry

NACA has multiple antioxidant properties. It is a well-known GSH prodrug and a free radical scavenger.[18-20] In cell-free systems, NACA can non-enzymatically restore GSH through thiol-disulfide exchange with the oxidized form, GSSG.[21] An annexin V-FITC and 7-AAD double staining assay was performed. Cells in the early apoptotic stages only bind annexin V-FITC, while cells in late apoptotic stages bind both annexin-V-FITC and 7-AAD. Dead/necrotic cells may only exhibit 7-AAD fluorescence, due to membrane disintegration and subsequent loss of phosphatidylserine. Table 1 summarizes the percent distribution of viable cells and cells in the early and late stages of apoptosis. The percentages of viable cells in the TBHP and NACA+TBHP groups are very similar (64.0 ± 4.9 % versus 67.8 ± 9.9 %). There was no significant difference in the number of dying cells (defined as the total of early- and late-stage apoptotic cells) between the TBHP and the NACA+TBHP groups (20.5 ± 6.1% versus 20.8 ± 4.8%). Because no significant improvement was observed in the cells pretreated with NACA, the dosing protocol was modified slightly without changing the total NACA exposure time (see Methods). Results of the modified treatment protocol are shown in Figure 6 and Table 2. In the NACA+TBHP (co-exposed) group, the percentage of viable cells (88.6 ± 0.1%) was significantly higher than that in the TBHP group (48.5 ± 5.1%, P<0.001). The percentages of dying cells in the TBHP group and the NACA+TBHP co-exposure group were 16.5 ± 3.6% and 5.5 ± 0.8%, respectively.

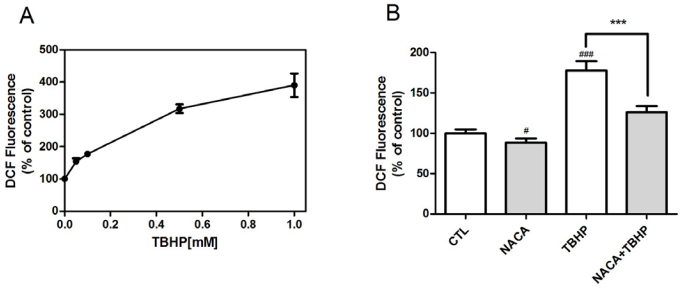

Figure 1: Intercellular ROS levels were measured using DCF fluorescence. (A) TBHP induced ROS generation in a dose-dependent manner. (B) NACA prevented TBHP-induced ROS generation.

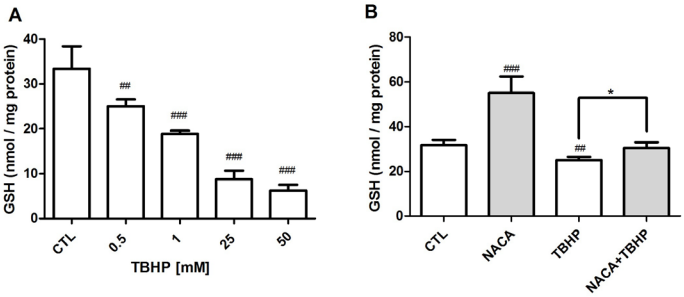

Figure 2: Intracellular GSH levels in HRPEpiC. (A) TBHP depleted GSH levels in a dose-dependent manner. (B) NACA dramatically increased intracellular GSH levels and prevented GSH depletion by TBHP.

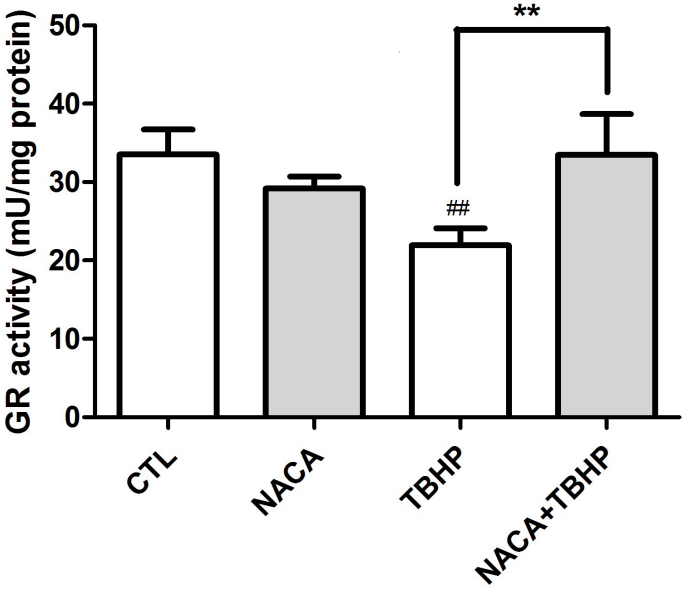

Figure 3: Preservation of glutathione reductase activity by NACA.

Table 1: Percent distribution of viable cells and cells in the early and late stages of apoptosis after 4 h pretreatment with NACA followed by 3 h TBHP exposure.

Groups	Control	2 mM NACA	0.5 mM TBHP	NACA+TBHP
Viable cells	94.8 ± 1.3	93.7 ± 0.5	64.0 ± 4.9	67.8 ± 9.9
Early apoptotic cells	1.3 ± 0.6	3.0 ± 1.1	1.8 ± 0.8	2.1 ± 0.4
Late apoptotic cells	1.9 ± 0.2	2.1 ± 0.5	18.8 ± 5.4	18.6 ± 4.9

The results are shown as mean % of total cells ± SD.

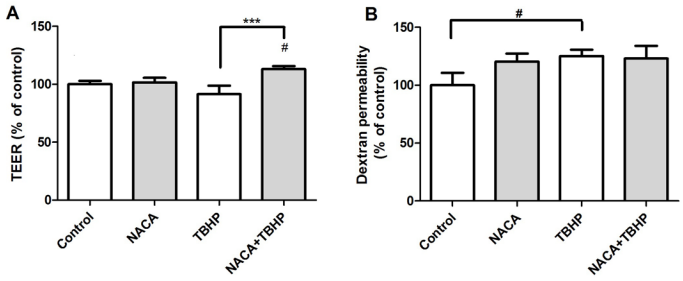

Figure 4: Protective effects of NACA on paracellular permeability in HRPEpiC. (A) Results of TEER measurement. (B) Results of dextran permeability assays.

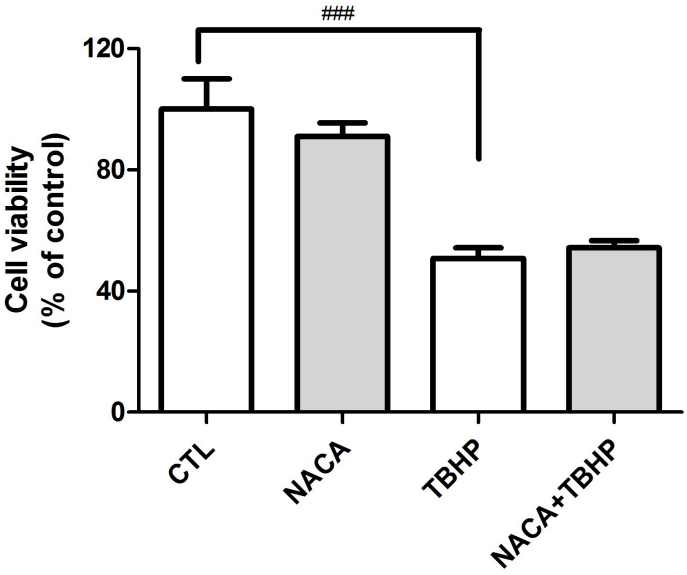

Figure 5: Effects of NACA and TBHP on HRPEpi cell viability.

Table 2: Percent distribution of viable cells and cells in the early and late stages of apoptosis from Figure 6.

Groups	Control	2 mM NACA	0.5 mM TBHP	NACA+TBHP
Viable cells	90.4 ± 1.2	84.8 ± 4.2	48.5 ± 5.1[a]	88.6 ± 0.0[b]
Early apoptotic cells	2.2 ± 0.3	1.7 ± 0.9	2.2 ± 1.0	2.3 ± 0.3
Late apoptotic cells	2.5 ± 0.4	4.4 ± 0.2	14.3 ± 3.5	3.1 ± 0.5

The results are shown as mean % of total cells ± SD. (a, $P < 0.001$ compared to control group; b, $P < 0.001$ compared to TBHP group).

DISCUSSION

In this study, we evaluated the potential of NACA to prevent oxidative stress-related damage *in vitro* in HRPEpiC. This allowed for evaluation of NACA as a defense against TBHP-induced oxidative injury. Selection of the best *in vitro* analogue to RPE in AMD patients was vital to the clinical relevance of our results. Currently, there are three cell models used to study the effects of oxidative stress on the RPE: ARPE-19, fetal human RPE cells (fhRPE) and primary culture adult human RPE (e.g., HRPEpiC). ARPE-19 is a spontaneously arising retinal pigment epithelial cell line derived from normal eyes. The fhRPE and HRPEpiC are primary cultures of fetal and adult human RPE cells, respectively. When

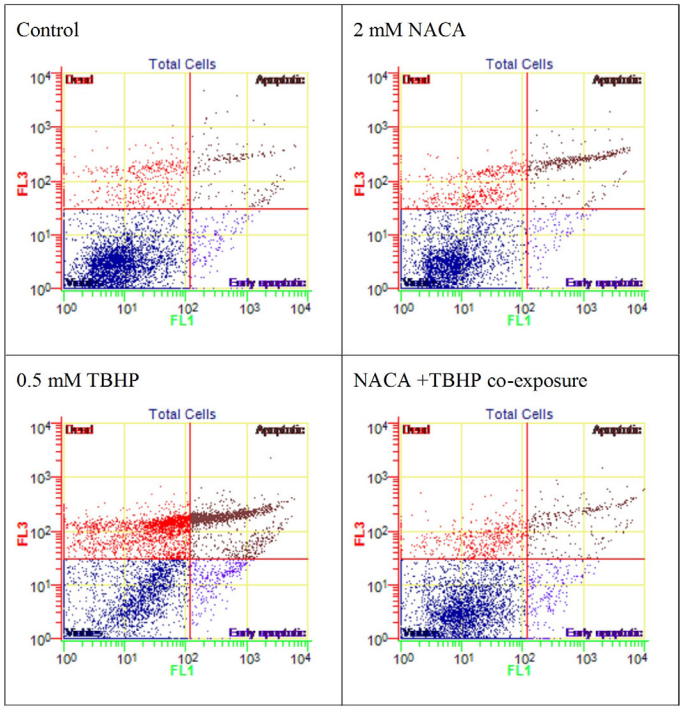

Figure 6: Flow cytometry analysis of apoptotic cells following the modified (co-exposure) protocol with NACA and TBHP. The dot plots show two-parameter analysis of fluorescence intensity of annexin-V FITC (FL1) and 7-AAD (FL3).

compared to ARPE-19, fhRPE cells are healthier; however, adult human RPE are more physiologically representative of the mature function of RPE than fhRPE.[22,23] Thus, an adult human primary RPE cell model was implemented for the experiments detailed here.

The RPE of the macula is particularly vulnerable to oxidative damage due to increased O_2 exposure, photo-oxidation, elevated metabolic activity, and high proportion of polyunsaturated fatty acids.[5] Our data demonstrates that TBHP increased ROS levels in a dose-dependent manner, leading to severe oxidative stress in the HRPEpiC cultures. NACA pre-incubation prevented TBHP-induced ROS production in HRPEpiC. It has been shown that accumulation of ROS induces severe oxidative stress in RPE cells, which is manifested by significant reduction in levels of GSH (the most critical antioxidant and redox buffer of the cell).[24,25] Results from our study showed a significant decrease in GSH levels in the HRPEpiC when compared to those of the control group (Figure 2). Pretreatment with NACA significantly increased GSH levels. This may indicate that NACA replenished GSH levels, leading to reversal of oxidative stress. The protective effects of NACA are probably mediated by a number of pathways, which may include supplying cysteine for GSH biosynthesis, reduction of extracellular cystine to cysteine, and conversion of GSSG to GSH by the action of GR and by non-enzymatic thiol disulfide exchange.[12,26-28] Our results are in agreement with previous studies which reported an increase in GSH levels after NACA incubation and a decrease in GSH levels upon TBHP treatment in an ARPE cell line.[11,14]

Importantly, changes in the GR activity were also observed. GR regenerates GSH from GSSG and plays an important role in GSH homeostasis.[29] NACA treatment increased the levels of GSH and restored GR activity (Figure 3). Our results are in line with another study that reported inhibition of GR activity in HepG2 upon TBHP treatment.[30]

In the retina, the RPE forms the blood-retinal barrier (BRB) with tight junctions that control the exchange of nutrients and metabolites between the retina and the underlying choriocapillaris. It is thought that oxida-

tive stress may induce changes in the BRB, which may contribute to the pathogenesis of retinal degeneration. To investigate the ability of NACA to preserve BRB function, membrane integrity after TBHP exposure was evaluated using dextran permeability and TEER assays.[31] NACA prevented TBHP-induced reduction in TEER, verifying its ability to protect cellular homeostasis and outer BRB integrity under severe oxidative stress conditions. A similar decrease in TEER has been reported in another study, where human embryonic stem cell (hESC)-derived RPE cells and ARPE-19 cells were exposed to hydrogen peroxide.[31,32] These results are also in agreement with a previous study performed in our laboratory using the ARPE-19.[14] However, we did not observe any significant difference in dextran permeability upon NACA treatment, which could be due to lower sensitivity of dextran permeability when compared with TEER.[33]

The decline in GSH observed creates a redox imbalance that enhances cell susceptibility to injury and ROS-induced apoptosis. Altering the redox state to a more reduced environment by addition of GSH has been shown to decrease sensitivity of RPE cells to apoptosis from ROS.[34,35] We initially investigated the protective effect of NACA pretreatment by studying cell viability. However, no significant improvement was noted in cell survival using either the Calcein-AM assay or flow cytometry. We therefore modified our study design to include co-exposure along with pre-incubation and dramatically improved cell survival (Figure 6). It is possible that pretreatment alone is not sufficient to address multiple oxidative stress-related apoptotic mechanisms. Oxidants, such as TBHP, may induce apoptosis through two different pathways: (1) Fas-Fas ligand activation and (2) mitochondrial damage and subsequent release of cytochrome c.[36] Because the interplay of these two pathways is complex, it is plausible that antioxidant treatments, while producing a more favorable redox status in the cell, may not fully address both apoptotic pathways. In addition, concentrations of TBHP (0.5 mM) and NACA (2 mM) chosen for our investigation may be too high and low, respectively, to observe significant cytoprotection by pretreatment with NACA. Our previous studies with ARPE-19 indicate that up to 10 mM NACA may be used to beneficial effect as a pretreatment for 0.4 mM TBHP exposure.[14]

CONCLUSION

This study demonstrates that treatment of retinal pigment epithelium with NACA protected against oxidative stress-induced cellular injury. NACA also inhibited oxidative stress-related breakdown of the blood-retinal barrier as measured by TEER. The proposed mechanism of action involves NACA scavenging existing ROS and increasing levels of reduced glutathione. With the prevalence of AMD and other retinal degeneration disorders expected to double in the coming decades, the development of more effective therapies to prevent its progression is imperative. Our results indicate that NACA has the potential to become an effective therapeutic agent by enhancing antioxidant defenses while removing pathologically relevant ROS.

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CONFLICT OF INTEREST

The authors have no financial, consulting, or personal conflicts of interest pertaining to this work.

ABBREVIATION USED

AMD: Age-related macular degeneration; **7-AAD:** 7-aminoactinomycin-D; **AREDs:** Age-related eye diseases; **GPx:** Glutathione peroxidase; **GR:** Glutathione reductase; **GSH:** Glutathione; **GSSG:** Reduces glutathione disulfide; **HRPEpiC:** Primary human retinal pigment epithelial cells; **NACA:** N-acetylcysteine amide; **NPM:** N-(1-pyrenyl)-maleimide; **ROS:** reactive oxygen species; **RPE:** Retinal pigment epithelium; **TBHP:** Tert-butyl hydroperoxide; **TEER:** Transepithelial electrical resistance.

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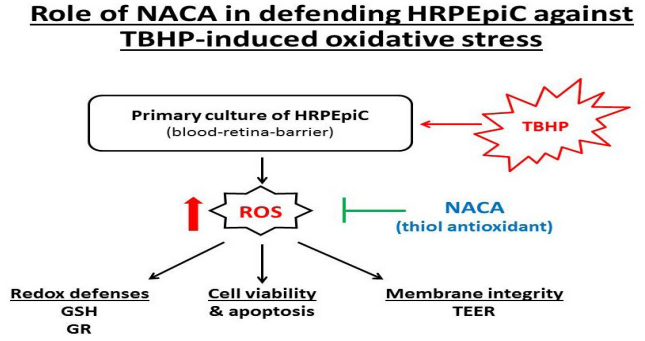
PICTORIAL ABSTRACT

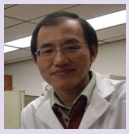


SUMMARY

- TBHP increased intracellular ROS levels in HRPEpiC, and NACA prevented ROS generation.
- Dose-dependent GSH depletion by TBHP was prevented by pretreatment with NACA.
- Pretreatment with NACA preserved of GR activity.
- NACA inhibited oxidative stress-related breakdown of HRPEpiC monolayer paracellular permeability.
- NACA significantly improved cell survival when administered prior to and during oxidative damage.

AUTHOR PROFILE

Hsiu-Jen Wang is a PhD candidate in the Department of Chemistry at Missouri University of Science and Technology (Missouri S&T). Her research focuses on the beneficial effects of NACA in oxidative stress-related macular degeneration. She also works on oxidative stress on muscarinic signaling pathways.



Dr. Yue-Wern Huang is currently a professor in the Department of Biological Sciences at Missouri S&T. Dr. Huang's research focuses on toxicity induced by nanoparticles, pharmaceuticals, and endocrine disruptors. He also specializes in targeted delivery mediated by cell-penetrating peptides and nanomaterials.

Dr. Shakila Tobwala obtained her Ph.D. from North Dakota State University and worked as a Scientist at Missouri S&T. She studied the effects of antioxidants in oxidative stress related disorders.

Annalise Pfaff is a PhD candidate at Missouri S&T who is interested in drug development for ocular disease and NMR investigations of biomaterials.

Dr. Robert Aronstam is the Dean of the College of Science and Technology at Bloomsburg University and a director of the cDNA Resource Center. His research interests are neurochemical and pharmacological characterization of muscarinic receptors and signaling. He is also interested in the effects of oxidative stress on muscarinic signaling pathways.

Dr. Nuran Ercal is the Richard K. Vitek/FCR Endowed Chair in Biochemistry at Missouri S&T. Her research examines the effects of free radicals and antioxidants in biological systems. In addition, her research group develops bioanalytical techniques for conducting investigations thereof.